# Optimization of SDS exposure on preservation of ECM characteristics in whole organ decellularization of rat kidneys

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**Abstract**

Renal transplantation is well established as the optimal form of renal replacement therapy but is restricted by the limited pool of organs available for transplantation. The whole organ decellularisation approach is leading the way for a regenerative medicine solution towards bioengineered organ replacements. However, systematic pre-optimisation of both decellularisation and recellularisation parameters is essential prior to any potential clinical application and should be the next stage in the evolution of whole organ decellularisation as a potential strategy for bioengineered organ replacements. Here we have systematically assessed two fundamental parameters (concentration and duration of perfusion) with regards to the effects of differing exposure to the most commonly used single decellularising agent (sodium dodecyl sulphate/SDS) in the perfusion decellularisation process for whole rat kidney ECM bioscaffolds, with findings showing improved preservation of both structural and functional components of the whole kidney ECM bioscaffold. Whole kidney bioscaffolds based on our enhanced protocol were successfully recellularised with rat primary renal cells and mesenchymal stromal cells to yield highly viable constructs. These findings should be widely applicable to decellularised whole organ bioscaffolds and their optimisation in the development of regenerated organ replacements for transplantation.

## Introduction

End stage renal failure is associated with major morbidity and mortality[1]. Renal transplantation is well established as the optimal form of renal replacement therapy (RRT) but is restricted by the limited pool of organs available for transplantation from deceased donors. According to the UK Renal Registry, there were 53027 adult patients receiving RRT on 31/12/2011 in the UK but the total number of kidney transplants performed in 2011 was 2752[1]. While a number of other approaches have been taken to attempt to artificially replace the functions of native kidney[2-8], recent innovations in the field of tissue engineering such as whole organ decellularisation may lead to a successful regenerative medicine approach and bioengineered solution to this pressing clinical need[9-11]. Whole organ decellularisation provides a relatively new approach to 'whole organ' engineering and this technique utilises the intrinsic vasculature to perfuse the decellularising agent and efficiently penetrate tissue and remove cellular material. The decellularised extracellular matrix (ECM) bioscaffold thus created preserves the intrinsic properties of the ECM[12] along with its natural organisational complexity in order to promote recellularisation and cellular differentiation on a whole organ level. The inherent vasculature is also critical in allowing perfusion to sustain cell culture in complex 3-D constructs. This approach has been successfully employed in the major solid organs to create viable and partially functional organ constructs, some of which have been implanted *in vivo*[13-18]. In particular recent work on the regenerated rat kidney has demonstrated up to 10% functionality in terms of urine production and creatinine clearance[18].

While the potential clinical implications are extremely exciting, a number of major technical factors and challenges exist before clinical application can be considered. One critical group of pre-optimisation parameters lies with the production of the ECM bioscaffold and the

decellularisation process. While the tissue- and organ-specific functional and bio-inductive molecules of the ECM are intrinsic to the success of the recellularisation and regenerative process, the quality of the ECM not only varies with each donor but also with each protocol applied[19]. A few studies have started to assess or compare effects of perfusion decellularisation protocols in a more systematic manner e.g. different decellularising agents[20], duration/exposure to the decellularising agent[21], but not in the rat kidney model. We have also shown in a recent systematic review that currently there can be significant disparity between decellularisation protocols in the literature even for the same organs/species, strongly suggesting the need for a more widespread evidence-based approach[22]. There are many parameters that exist to be considered – such as choice of decellularising agent (which also depends on type of tissue being decellularised), concentration and duration of perfusion (i.e. exposure) of decellularising agent on the tissue/ECM, additional steps in the protocol (e.g. freezing, use of biological agents), tissue harvesting factors, perfusion versus non-continuous, perfusion pressure versus perfusion rate, and last but not least, post-processing sterilisation methods.

On this basis, we aimed to optimise two fundamental parameters (i.e. concentration of decellularising agent and duration of perfusion) in order to assess the effects of differential exposure to decellularisation agent for whole rat kidney ECM bioscaffolds, using the most commonly reported single decellularising agent i.e. sodium dodecyl sulphate (SDS)[22]. Optimisation was determined with regards to structural and functional characteristics of the ECM bio-scaffold as assessed by histology, immunohistochemistry (IHC), quantitative assays of DNA and sulfated glycosaminoglycan (sGAG) content, and growth factor quantification – and optimisation was continued until a 'plateau' effect ('no further comparative benefit'?) was observed in one or more of these modalities. We have also performed perfusion-based

recellularisation of these whole rat kidney ECM bioscaffolds with rat primary renal cells and mesenchymal stromal cells to demonstrate high levels of cellular engraftment and cellular viability with *in vitro* perfusion culture, and formation of renal-like structures on histology.

## Methods and materials

### Decellurisation of whole kidneys

1. Tissue harvesting

   Male Wistar rats (350-375 g, Charles River/UK) were chosen for kidney harvest for perfusion decellularisation. Briefly, renal arteries were cannulated immediately after euthanisation of the animal and perfused with phosphate buffered saline (PBS) with vasodilator (10 ml of 10 μg/ml sodium nitroprusside in PBS, Sigma/UK, followed by 20 ml at 1 μg/ml) until a uniform blanching was observed, after which each kidney was perfused with 30 ml PBS without vasodilator.

2. Whole kidney decellularisation

   Kidneys were suspended within a reservoir and perfused continuously via the arterial cannula at 10 ml/min, recirculating the total volume of 400ml of decellularising solution throughout the decellularisation process. The perfusate was sodium dodecyl sulfate (Sigma/UK) at differing concentrations and durations (see Tables 1a + 1b). After decellularisation, kidney bioscaffolds were perfused with PBS for 1 hour. Those for subsequent recellularisation were sterilised with 1% w/v antibiotic-antimycotic (Gibco®, Invitrogen/UK) in sterile PBS for 18 hours at 2 ml/min.

3. Histology and immunohistochemistry (IHC)

All histological samples were fixed in 10% v/v buffered neutral formalin and stained with haematoxylin and eosin (H+E) for light microscopy.

IHC was performed to assess retention of key ECM and basement membrane proteins collagens I and IV, laminin and fibronectin (all Abcam/UK). All samples were treated identically with regards to fixation, antigen retrieval, antibody staining, blocking and development. Briefly, paraffin-embedded sections were dewaxed and blocked with 1% v/v hydrogen peroxide in methanol for 10 minutes. Heat-mediated antigen retrieval was performed in 0.01M sodium citrate buffer (Sigma/UK), pH 6.0, followed by Avidin-Biotin blocking (Dako/UK). Sections were blocked for 1 hour with 5% v/v goat serum in PBS, and primary antibodies were incubated overnight at 4$^{o}$C. Primary antibody dilutions in blocking solution (5% v/v goat serum in PBS) were: collagen I 1:250, collagen IV 1:500, laminin 1:50, and fibronectin 1:500 (Abcam/UK). After washing sections in 0.1% v/v TWEEN (Sigma/UK) in PBS, pH 7.4, hydrogen peroxidase conjugated secondary antibody (Abcam/UK) was applied at 1:100 for 1 h at room temperature then washed again in TWEEN solution. Slides were developed using 3,3'-diaminobenzidine (Dako/UK) until good staining intensity was observed. Species immunoglobulin and PBS served as negative controls. Haematoxylin was used for nuclear counterstaining.

4. <u>DNA/sGAG quantification</u>

Approximately half of a whole, unfixed rat kidney or kidney bioscaffold was suspended within 5 ml of papain solution (55 mM L-cysteine hydrochloride, 80 mM EDTA, 2.5 units/ml papain, pH 6.0, all Sigma/UK) for 24 h at 60$^{o}$C until no visible solid material remained. After digestion, the solution was freeze-dried for 24h using the

Heto PowerDry LL1500 freeze dryer (Thermo Scientific/UK) to ascertain the dry weight.

Total DNA content was quantified using the Picogreen® dsDNA assay (Invitrogen/UK) and total sGAG content was quantified using the Blyscan™ sGAG assay (Biocolor/UK) according to the manufacturer's instructions. Each assay was performed in duplicate and three times in total.

5. Growth Factor extraction and quantification assays

Approximately half of a whole, unfixed rat kidney or kidney bioscaffold was suspended in ~3.75 ml of urea–heparin extraction buffer (2 M urea and 5 mg/ml heparin in 50 mM Tris with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM N-ethylmaleimide at pH 7.4, all Sigma/UK). The extraction mixture was rocked at 4°C for 20-24 h then centrifuged at 5,000 x *g* for 30 min; supernatants were collected. 6 ml of extraction buffer was added to each pellet. These were again rocked at 4°C for 20-24 h, centrifuged at 5,000 x *g* for 30 min, and supernatants collected. Supernatants from both extractions were dialyzed against ultrapure filtered water (total of three changes of dialysis water, 80-100 volumes/change) in Spectra/Por 3500 MWCO dialysis membrane (Spectrum Lab./USA). Extracts were frozen in aliquots at -80˚C until assayed. Total protein concentration in each dialyzed extract was determined by the BCA protein assay (Pierce, Thermo Scientific/UK) following the manufacturer's protocol. Basic fibroblast growth factor and vascular endothelial growth factor concentrations were determined with the Quantikine Human FGF basic Immunoassay and Quantikine Rat VEGF Immunoassay (R&D Systems/UK) respectively, following the manufacturer's protocols. Each growth factor assay was performed in duplicate three times.

It should be noted that each growth factor assay measured growth factor protein concentration and did not measure growth factor activity.

6. Vascular resin corrosion casting

   A 0.5-1.0 ml volume of polymer mixture, depending on the kidney size, was injected via the arterial cannula using Batson's no. 17 anatomic corrosion casting kit (Polysciences Inc./USA) as recommended by the manufacturer. Polymerisation took 20-30 min at 4°C and photographic images were taken during the injection process over 10-30 seconds.

7. Statistical analysis

   Results were reported as mean +/– standard error. Statistical analysis was performed with analysis of variance which demonstrated non-parametric distribution of data followed by Mann Whitney two sample test. A *p*-value of $< 0.05$ was considered significant.

**Recellularisation of the whole kidney bioscaffold**

1. Recellularised kidney graft perfusion – system setup

   After sterilisation, the decellularised kidney bioscaffold was seeded with 50 x $10^6$ cells in divided multistep bolus injections via the arterial cannula at 45-60 minute intervals. Typically three injections of approximately 16 x $10^6$ cells at 32 x $10^6$ cells/ml were used. The recellularised kidney bioscaffold was then suspended in the main chamber of a sterile three-port flask immersed within the reservoir of culture medium (150-180 ml volume). The perfusion system also included a sterile exchange membrane (BugStopper, Whatman/UK) and a peristaltic pump. The system was placed inside a standard 37°C 5% $CO_2$ incubator for temperature and $CO_2$ control.

The graft was continuously perfused at 1.5 ml/min. The culture medium was changed every 48 h throughout the 7 day culture period (the first change occurring at 24 hours post-seeding).

The perfusate consisted of either i) 50% DMEM and 50% Keratinocyte Serum-Free Medium (K-SFM) with bovine pituitary extract (BPE) and epidermal growth factor (EGF) with the addition of 5% v/v foetal bovine serum (FBS) and 1% v/v antibiotic-antimycotic (AA) for primary renal cells, or ii) $\alpha$-MEM with the addition of 10% v/v FBS and 1% v/v AA (all Gibco®, Invitrogen/UK) for mesenchymal stromal cells.

2. Primary renal cell (PRC) isolation

Four-week-old Wistar rats were used for PRC isolation (protocol adapted from Joraku *et al*[16]). Briefly, minced kidney tissue from culled animals was digested in collagenase solution (Liberase TM Research Grade, Roche/UK) according to the manufacturer's recommendations. The digested tissue suspension was filtered through a 100 $\mu$m cell strainer (BD Falcon®, Corning/UK) and neutralised with an equal volume of DMEM culture medium. This solution was then centrifuged at 1200 rpm for 5 minutes. The resulting cell pellets were re-suspended and plated in 50% DMEM + 50% K-SFM with BPE/EGF with the addition of 5% v/v FBS and 1% v/v AA. PRCs were passaged when they reached 95% confluence and typically used for recellularisation at P2 stage.

3. Mesenchymal stromal cell (MSC) isolation

Male Wistar rats (350-375g) were used for MSC isolation. Briefly, sterile femurs from culled rats were irrigated (by manual injection) through the diaphysis with culture medium ($\alpha$-MEM) and the resulting cell suspension plated in $\alpha$-MEM with 10% v/v

FBS and 1% v/v AA. MSCs were passaged when they reached 95% confluence and typically used for recellularisation at the P2 stage.

4. LIVE/DEAD™ staining

   The tissue sample was washed with PBS, and then completely immersed in the working solution (20 μl of Ethd-1 and 10 μl of calcein (Invitrogen/UK) in 10 ml of sterile PBS). This solution was incubated with the tissue sample for a period of 10-15 minutes while being protected from light, then aspirated. The sample was washed three times with PBS and examined using fluorescent microscopy.

5. AlamarBlue® assay

   A working solution of 1:10 alamarBlue® reagent (Invitrogen/UK) in culture medium was added directly to the tissue to completely immerse the sample. Samples and controls (working solution without tissue sample) were incubated for 4 hours at 37°C in 5% $CO_2$ incubator**.** Each assay was performed in triplicate. Results were reported as mean +/– standard error.

6. Histology and IHC

   All samples were fixed in 10% v/v buffered neutral formalin, and samples were stained with haematoxylin and eosin (H+E) for light microscopy.

   IHC was performed to characterise cell types and their distribution on the recellularised kidney bioscaffolds; the protocol is the same as above. Primary antibody dilutions were: aquaporin-1, aquaporin-2, synaptopodin, and von Willebrand Factor (all Abcam, UK) – all at 1:500.

For all experimental work as described above, $n \geq 3$.

## Results

### Optimisation of perfusion decellularisation in whole rat kidneys

We have decellularised cadaveric whole rat kidneys using perfusion decellularisation via the renal artery with SDS initially at 1% concentration performed for decreasing durations of continuous perfusion (24 down to 4 hours) at 10 ml/min with 1% w/v SDS (Table 1a), which all yielded well decellularised bioscaffolds on histological analysis (Supplementary Fig. 1). Decellularisation was characterised by: histology and IHC, DNA and sGAG quantification, and growth factor (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) quantification. The 1% w/v SDS x 4 hours protocol was denoted the baseline protocol for further comparisons. Two further series of protocols were carried out in decreasing concentrations of SDS (0.5%, 0.25%, and 0.125% w/v) and for two durations of perfusion (4 hours and 8 hours, Table 1b). All further decellularisation protocols were characterised similarly to the baseline protocol.

*Histology and IHC*

Histological analysis of perfusion decellularised kidney bioscaffolds shows preservation of the structure and architecture of the renal ECM within the acellular parenchyma (Fig. 1), as has been previously shown[18, 23, 24]. The preservation of the arterial network within the whole kidney bioscaffolds was also demonstrated with injection of a resin polymer for vascular casting (Fig. 2). Immunohistochemical staining for key ECM components (collagen I, collagen IV, laminin and fibronectin) shows a physiological distribution as compared to the normal kidney tissue control (Fig. 1). There were no gross differences observed between any of the protocols (variation in % SDS or duration of perfusion) in terms of the histological

appearance and IHC staining (see also Supplementary Fig. 2 for full range of conditions analysed).

*Quantification Assays*

For all protocols, DNA levels in the decellularised kidney bioscaffolds (Fig. 3c) were ≤0.02% of normal kidney DNA content. Quantification of sGAG levels (Fig. 3a, b) show an at least two-fold increase in the average levels of sGAG preserved between the 4 hour and 8 hour protocols ($p < 0.005$).

Levels of VEGF and bFGF preserved within the kidney ECM (Fig. 3d-f, g-i) were similar in that the 8 hour levels for both growth factors were significantly decreased compared to both normal kidney and the 4 hour protocols, and were in fact almost negligible for both. However, the levels of both growth factors were well preserved compared to normal kidney in the 4 hour series (3d-e, g-h), with the highest levels of preservation seen at the lowest concentration of SDS exposure i.e. 0.125% w/v in both VEGF and bFGF ($p < 0.05$). A clear trend of increasing levels of bFGF preserved as the concentration of SDS decreases can also be seen; however a plateau effect can be observed with both VEGF and bFGF as the difference observed between levels with 0.25% w/v SDS and those with 0.125% w/v was not significant ($p = 0.256$ for both). Group variations were noted in the levels obtained for all the quantification assays which are likely to be due to batch variation.

**Recellularisation of whole kidney bioscaffolds**

To recellularise the acellular whole kidney bioscaffolds for *in vitro* culture, these were injected intravascularly with two cell populations in parallel studies: primary renal cells (PRCs) isolated from 4 week old Wistar rats and adult Wistar rat bone marrow-derived

mesenchymal stromal cells (MSCs). After the cell seeding process, perfusion was maintained at 1.5ml/min for the designated 7 day culture period.

*PRC-recellularised constructs*

Cellular viability tests (Fig. 4a, b) after 7 days of *in vitro* culture demonstrate continued and extensive viability throughout the 3-D kidney construct, with obvious cellular 'patterning' following the vascular network and also ECM structural cues on LIVE/DEAD™ staining.

Histological analysis showed cellular engraftment dispersed throughout the kidney ECM bioscaffolds which was not restricted to the vascular compartment. Focal areas of recellularisation comparable to normal histological density were present throughout and distinctive 'glomerular-like' structures are easily observed (Fig. 5a). IHC of renal cell markers (aquaporin 1 and aquaporin 2 for tubular cells, synaptopodin and von Willebrand factor for glomerular epithelial and endothelial cells respectively) demonstrated the presence of mixed cell types throughout the recellularised construct (Fig. 5b).

*MSC-recellularised constructs*

Cellular viability tests (Fig. 4a, b) after 7 days of *in vitro* culture were similar to the PRC constructs: extensive viability throughout the 3-D kidney construct, with obvious cellular 'patterning' following the vascular network and also ECM structural cues on LIVE/DEAD™ staining. Histological analysis again showed cellular engraftment dispersed throughout the kidney ECM bioscaffolds with focal areas of recellularisation comparable to normal histological density present throughout and distinctive 'glomerular-like' structures (Fig. 5a). IHC of renal cell markers (as above) were only strongly positive for von Willebrand factor as an endothelial cell marker (Fig. 5b).

## Discussion

Various groups have demonstrated the feasibility of using the whole organ decellularisation approach to derive bioengineered (partially) functional organ constructs for surgical implantation[14-18]. While there is great interest in the recellularisation potential of these bioscaffolds, this potential should be maximised by optimisation of the quality of the ECM preserved after perfusion decellularisation. Both donor factors and decellularisation parameters should be standardised and rationalised to yield consistent and optimised bioscaffolds. Pre-optimisation and standard operating procedures are even more critical when it is likely that post-processing testing of the ECM bioscaffold (and the recellularised construct) is likely to be limited in scope in those cases destined for implantation. This should be the next stage in the evolution of whole organ decellularisation as a potential strategy for bioengineered organ replacements.

Currently, most groups are utilising disparate decellularisation protocols with marked variations even in work on the same organ and species[17, 25-29]. Hence we present here an evidence-based approach to determining a more systematically assessed protocol for whole rat kidney bioscaffolds using a single agent (SDS) with continuous perfusion, as defined by preservation of essential ECM components within the bioscaffold. The choice of SDS was derived from a systematic review of the whole organ decellularisation literature and SDS being the pre-dominant decellularising agent of choice, sometimes in combination with Triton-X100 or other agents[22]; SDS was utilised alone within this study as it has been well documented to produce complete decellularisation within ECM literature and also for purposes of clarity.

SDS ($CH_3(CH_2)_{11}OSO_3Na$) is an ionising detergent with molecule amphiphilic properties and critical micelle concentration (CMC) is 8.2 mM or approximately 0.23% w/v[30]; it is not pH-

sensitive between pH 5-10. It acts by solubilising cell membranes and nucleic membranes, with very effective removal of nuclear remnants and cytoplasmic proteins. However it can also denature proteins, remove GAG and growth factors, and cause damage to collagen and the ultrastructure of basement membranes[19] – which could have subsequent detrimental effects on recellularisation. Whole organ decellularisation with its underlying technique of vascular perfusion lends itself well to the principle of minimising SDS exposure since the native vascular system allows the maximum efficiency in decellularising agent delivery and penetration, especially to dense tissues. Historical protocols traditionally utilising SDS at 1% or even higher are not necessarily relevant in this context. Indeed, concentrations above the CMC lead to micellar formation of SDS molecules and subsequent plateauing of increased detergent efficacy. In addition, SDS residuals may be highly adherent and cytotoxic (with subsequent detrimental effects on recellularisation), and hence it would be important for these to be quantified in future work e.g. quantification assays of solubilised decellularised scaffolds using a colorimetric assay method [31]. Currently this is a significant limitation of all extant whole organ decellularisation work utilising SDS.

Our results have yielded an improved protocol (0.125% w/v SDS perfused for 4 hours) which uses a far lower concentration of SDS than other work on whole rat kidney decellularisation[18, 23] while being at least as effective in achieving removal of cellular components and DNA. The minimum concentration of SDS previously utilised for whole rat kidney decellularisation was typically 1%[18], although one study has used 0.5% in porcine kidneys[24], and the concentration can be variable up to as high as 4%[23]; no explicit justification (or systematic comparative characterisation of decellularised scaffolds) for any specific conditions have been stated in any study[18, 23, 24] except for *de facto* demonstration of 'successful decellularisation'.

VEGF and bFGF are both highly expressed within the kidney and are vital to both cellular proliferation and kidney development. VEGF is expressed by virtually every endothelial cell within the kidney[32], which is particularly relevant to the highly intricate vasculature of the renal nephron; bFGF is involved in critical functions such as mitogenesis[33]. These two growth factors were selected for their importance and ubiquity within the kidney for quantitative assessment. Here we have also demonstrated a clear relationship between decreasing exposure to SDS (i.e. concentration) and preservation of *in situ* growth factors, down to the level where further decreases in the SDS concentration may not yield significantly increased preservation, which has not been previously shown for any whole organ bioscaffolds.

In line with other kidney studies[18, 23], after confirmation of successful decellularisation, we have recellularised the whole rat kidney bioscaffolds (by intravascular injection) with PRCs and continuous *in vitro* perfusion and culture for 7 days, leading to viable constructs. Histological analysis showed focal areas of dense cellular engraftment with some response to kidney ECM structural cues although IHC analysis did not show appropriately localised glomerular or tubular cells. We have also achieved comparable findings in recellularisation of whole rat kidney bioscaffolds with MSCs, as they are a primary cell population of relevance in potential clinical application. MSCs have been observed to have nephrogenic potential[34-37] and as they are both sufficiently numerous in the adult and a potentially host-derived (and hence immune-tolerant) cell population, they may play a crucial role in the clinical translation of bioengineered whole organ constructs. As there was no evidence of renal cellular differentiation with histological analysis, we did not proceed with renal functional testing. However, since Song *et al* recently demonstrated urine production *in*

*vitro* with their recellularisation protocol[18], it is possible that recellularisation with PRCs can lead to potentially functional renal regeneration on a whole kidney bioscaffold.

While we have examined here two fundamental parameters within the decellularisation process using SDS for whole rat kidneys, there are many other parameters of significance employed within decellularisation protocols as stated before – such as other decellularising agents (relevant to the type of tissue in question), additional processes e.g. freezing, use of additional agents, tissue harvesting factors, perfusion factors, and post-processing sterilisation methods[22]. All these will inevitably affect the quality of the ECM bioscaffold to a greater or lesser degree, and these effects have yet to be systematically quantified for whole rat kidneys or other organs/species. In addition, while growth factor levels may be better preserved by reducing the concentrations of the decellularising agent used (as we have demonstrated), functional tests of bioactivity should also be considered for more complete characterisation, such as comparative recellularisation studies related to different decellularisation parameters[20]. This is another current limitation and major area for future improvement. All these factors should play a critical part in developing safe, reliable and consistent whole organ bioscaffolds if they are to be taken to the clinical stage in the development of regenerated organ replacements.

In this study, systematic assessment of perfusion decellularisation parameters for whole rat kidneys with SDS has led to improved preservation of sGAG and growth factors along with minimised exposure to the decellularising agent and its detrimental effects; these whole rat kidney bioscaffolds were successfully recellularised with primary renal cells and MSCs to yield highly viable constructs. These findings may be applicable to other decellularised whole organ/species, and may lead the way to further optimisation and standardisation in the development of regenerated organ replacements for transplantation.

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